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Technical Note

Biotransformation of benzothiazole derivatives by the *Pseudomonas putida* strain HKT554

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ABSTRACT

We examined the biotransformation of benzothiazole derivatives (BTHs) by an axenic microbial culture. A Gram-negative bacterium, tentatively named as strain HKT554 and identified as *Pseudomonas putida*, was able to transform not only benzothiazole and 2-mercaptobenzothiazole but also 2-methylthiobenzothiazole, which was previously reported as the dead-end product of wastewater treatment. GC/MS analysis of the solid-phase extract of the culture broth showed the formation of 2-(3H)-benzothiazolone/2-hydroxybenzothiazole from benzothiazole. By transposon mutagenesis, a mutant library containing ca. 5000 insertion mutants was constructed from the *P. putida* strain HKT554. Analysis of the disrupted gene from one of the mutants showing BTHs transformation deficiency revealed that the knocked-out gene was naphthalene dioxygenase. To our knowledge, this is the first report on the biotransformation of BTHs by Gram-negative bacteria.

1. Introduction

Benzothiazole derivatives (BTHs) are a group of xenobiotic heterocyclic chemicals that contain a benzene ring fused with a thiazole-ring. BTHs are manufactured worldwide for a wide variety of applications. They are mainly used as vulcanizing agents in the rubber industry (De Wever and Verachtert, 1997), as fungicides in lumber and leather production (Genschow et al., 1996), as corrosion inhibitors in antifreeze, in industrial cooling systems (Reddy and Quinn, 1997). These applications clearly indicate that BTHs have a wide spectrum of biological activity. Released from factories producing and using BTHs, these compounds have been detected in industrial wastewaters (Kloepfer et al., 2005) and in various environmental compartments, such as soil, groundwater, and surface water, via drainage systems (Catallo and Junk, 2005). The limited biodegradability of BTHs, their potential toxicity toward microorganisms (De Wever et al., 1994), their allergenicity (Chipinda et al., 2007), and their potential mutagenic effects (Gold et al., 1993) make their presence in the environment a great concern.

Few researchers have reported on the biodegradation of BTH and then only with Gram-positive strains represented by *Rhodococcus* spp. De Wever et al. (1998) reported *Rhodococcus erythropolis* is able to degrade 2-hydroxybenzothiazole, benzothiazole-2-sulfonate, and BTH but not 2-mercaptobenzothiazole (MBT). Biodegradation pathways of BTH, 2-hydroxybenzothiazole, and MBT have been partially elucidated with the *Rhodococcus pyridinovorans* strain PA (Haroune et al., 2002) and the *Rhodococcus rhodochrous* strain OBT18 (Haroune et al., 2004). The degradation of 2-aminobenzothiazole by *R. rhodochrous* was recently reported (Bunescu et al., 2008a,b; Chorao et al., 2009). In addition, MBT, which was considered as one of the most utilized compounds for the vulcanization accelerator synthesis in the rubber industry, was reported to convert into 2-methylthiobenzothiazole (MTBT) as the dead-end product of wastewater treatment (Reemtsma et al., 1995).

In our previous study, *Pseudomonas* sp. HKT554 was able to degrade not only polyaromatic hydrocarbons, such as naphthalene and phenanthrene, but also aromatic heterocycles, such as naphtho [1,2-*b*] thiophenes, dibenzothiophene, alkylated benzothiophene, and dibenzofuran (Matsui et al., 2003). Although the degradation for some of them were shown to be catalyzed by the naphthalene dioxygenase (NDO) system, there have been no reports if NDO could be involved in BTH degradation. In addition to the wide substrate specificity of NDO, it is of interest with regard to the fate of the BTHs by such an enzyme system since it catalyzes not only aromatic ring hydroxylation but also the branched chains and heterocycle oxidation (Resnick et al., 1996).

Here, we report the transformation of BTH, MBT, and MTBT by the Gram-negative bacterium *Pseudomonas putida* strain HKT554.

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2. Materials and methods

2.1. Chemicals

Benzothiazole (Fig. 1a), MBT (Fig. 1b), and MTBT (Fig. 1c) were supplied by Wako Pure Chemicals Co., Osaka, Japan. 2-(3H)-benzothiazolone (Fig. 1d) was provided by Tokyo Kasei Kogyo Co., Tokyo, Japan. All the other materials were of the highest purity, commercially available, and used without further purification.

2.2. Bacterial strain and growth conditions

The *Pseudomonas* sp. strain HKT554 was used (Matsui et al., 2003). It was cultivated aerobically in Luria–Bertani (LB) medium (10 g bacto-tryptone, 5 g yeast extract, 10 g NaCl in 1000 mL deionized water) at 30 °C. To prepare a resting cell suspension, cells cultured in LB medium at late logarithmic phase were washed twice with sterilized deionized water and once with 67 mM sodium phosphate buffer (pH 7.0) and resuspended in an appropriate volume of the phosphate buffer to adjust the cell concentration to an optical density at 660 nm of 10, as described previously (Matsui et al., 2003). The cell suspension was treated at 121 °C for 5 min in case of heat-inactivated cells to examine the possibility of substrates adsorption to the resting cells. The control used in this experiment was resting cell suspension inoculated with BTH derivatives without bacterial cell. The reaction was started by adding the substrate. BTH compounds were added to 1 mL of the cell suspensions as *N,N′*-dimethyl formamide (DMF) solution at a final concentration of 0.1 mM. The blank used in this experiment was the resting cell suspension. DMF was also included in blank tests since it is used to dissolve the BTHs. All experimental data are the means of duplicate measurements, with the sampling frequency of 5 h. The incubation conditions were: 30 °C, aerobic, shaker speed: 180 rpm. Medium A (Izumi et al., 1994) containing 10 g L^{-1} glucose and 0.5 g L^{-1} $MgSO_4 \cdot 7H_2O$ as the sole carbon and sulfur source, respectively, was also used as the minimal medium. All tests were carried out in duplicate.

2.3. Analysis

Growth of bacterial cells was estimated spectrophotometrically by measuring the optical density at 660 nm. Substrate consumption was analyzed with the supernatant obtained from the reaction mixture by centrifugation at 4 °C, 15 000 rpm for 5 min using high performance liquid chromatography (HPLC) (Type LC-10A, Shimadzu Co., Kyoto, Japan) under ultraviolet detection set at 254 nm with a column for reverse-phase analysis (Type VP-ODS Shim-pack, 150 mm × 4.6 mm, Shimadzu Co., Kyoto, Japan). HPLC running conditions were as follows for the mobile phase: methanol/water = 70/30; flow rate = 1 mL min^{-1}. Accumulating product was recovered by using a solid-phase extraction method (Oasis from Waters Co., MA, USA) with methanol for the elution, carried out according to the manufacturer's instructions, followed by using it for GC/MS and HPLC/MS analysis. GC/MS analysis was performed as described previously (Matsui et al., 2010). Structure assignments were confirmed using the National. Bureau of Standards registry of mass spectral data (John Wiley, NY, USA) as previously described (Matsui et al., 2001b). HPLC/MS analyses were performed using the Agilent 1100 analytical HPLC system. The HPLC conditions were as follows mobile phase: methanol/water = 70/30; flow rate = 1 mL min^{-1}; injected volume = 1 μL with a VP-ODS column (Shim-pack, 150 mm × 4.6 mm, Shimadzu Co., Kyoto, Japan). The HPLC system described above was combined with a Bruker Esquire 3000 ion trap mass spectrometer (Bruker-Franzen Analytik GmbH, Bremen, Germany) equipped with an ESI interface. Instrument control and data acquisition were performed using Esquire 5.0 software. MS conditions: positive ion mode; drying gas (N_2) flow, 10 L min^{-1}; temperature: 350 °C; nebulizer gas (N_2) pressure, 40 p*K*a; capillary voltage, +1.5–4.5 kV; cone voltage, 15–100 V; scan range: 50–300 *m/z*; scan resolution: 13 000 *m/z* s^{-1}.

2.4. DNA manipulations and molecular genetic protocols

EZ: TN Tnp < KAN-2 > Transposome Kit (EPICENTRE Biotechnologies Co., Madison, WI, USA) was used to obtain transposon mutants of the HKT554 strain, according to the manufacturer's instructions. Transposon mutagenesis was performed by introducing Tnp < KAN-2 > into HKT554-competent cells by electroporation, as described by Choi et al. (2006). The cells of strain HKT554 competent for electroporation were prepared by cultivating 50 mL of cells in 200 mL flask in LB medium. The flask was incubated at 30 °C with shaking until the culture reached the exponential phase. Then, cells were centrifuged for 2 min at 4 °C and 8000 rpm. The cell pellet was washed twice with 300 mM sterilized sucrose solution, suspended in 0.8 mL of 300 mM sucrose solution, dispensed as 0.1 mL for each microtube and centrifuged. The cells were finally suspended in 100 μL of 10% (w/v) glycerol solution and stored at −80 °C until used. Competent cells (100 μL) and 1 μL transposon were mixed and electroporated at 2.5 kV, 25 μF, 200 Ω, and 0.2 cm cubet using MicroPulser electroporator (Bio-Rad, Hercules, CA, USA). Obtained clones grown on LB plates containing 100 mg L^{-1} kanamycin (Km) were used for

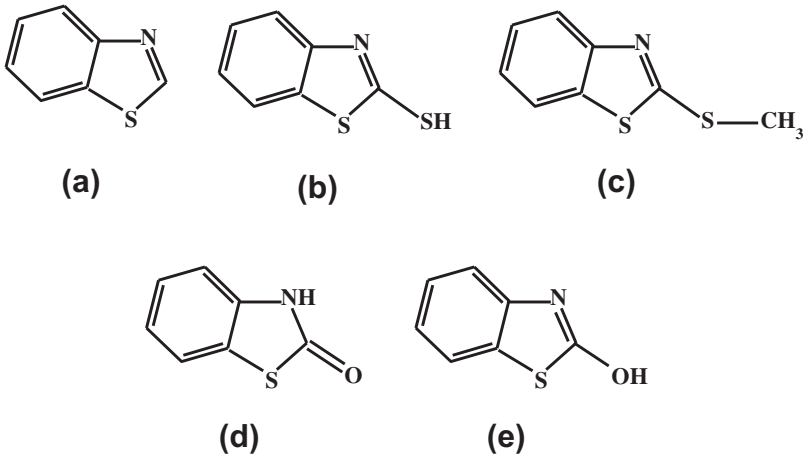

Fig. 1. Chemical structures of benzothiazole derivatives (BTHs) used in this study: (a) benzothiazoles, BTH; (b) 2-mercaptobenzothiazole, MBT; (c) 2-methylthiobenzothiazole, MTBT; (d) 2-(3H)-benzothiazolone; (e) 2-hydroxybenzothiazole.